Occurrence of Resistance-Breaking *Beet necrotic yellow vein virus* of Sugar Beet

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ABSTRACT

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Rhizomania is an important virus disease of sugar beet and is caused by *Beet necrotic yellow vein virus* (BNYVV). During 2002–03, several sugar beet fields with cultivars partially resistant to BNYVV grown in the Imperial Valley of California were observed with severe rhizomania symptoms, suggesting that resistance conditioned by *Rz1* had been compromised. Soil testing with sugar beet baiting plants followed by enzyme-linked immunosorbent assay (ELISA) was used to diagnose virus infection. Resistant varieties grown in BNYVV-infested soil from Salinas, CA, were ELISA-negative. In contrast, when grown in BNYVV-infested soil collected from the Imperial Valley, CA, all resistant varieties became infected and tested positive by ELISA. Based on host reaction, eight distinct BNYVV isolates have been identified from Imperial Valley soil (IV-BNYVV) by single local lesion isolation. Reverse transcription–polymerase chain reaction (RT-PCR) assays showed that the eight IV-BNYVV isolates did not contain RNA-5. Single-strand conformation polymorphism banding patterns for the IV-BNYVV isolates were identical to A-type and different from P-type. Sequence alignments of PCR products from BNYVV RNA-1 near the 3′ end of IV-BNYVV isolates revealed that both IV-BNYVV and Salinas BNYVV isolates were similar to A-type and different from B-type. Our results suggest that the resistance-breaking BNYVV isolates from Imperial Valley likely evolved from existing A-type isolates.

Rhizomania is one of the most economically important diseases of sugar beet (*Beta vulgaris* L.) and is widely distributed in most sugar beet growing areas worldwide. The disease is caused by *Beet necrotic yellow vein virus* (BNYVV) (24,25) and vectored by the plasmodiophorid *Polymyxa betae* Keskin (6). Most sugar beet production areas are dependent upon resistant sugar beet cultivars to control this devastating disease (3). Sources of resistance to rhizomania were found in the USDA-ARS sugar beet breeding programs at Salinas, CA (3,14,16). Resistance to BNYVV in the Holly Sugar Company's germ plasm is inherited as a single dominant allele (*Rz1*) (3,16). The number of alleles in a genotype and ratio of *Rz1* to *rz1* alleles in a cultivar are important in the overall performance of sugar beet cultivars under conditions favorable to rhizomania infestation (27). Resistance to BNYVV has also been obtained from several wild beet (WB) accessions of *B. vulgaris* subsp. *maritima* (14). Resistance in WB42, originally collected in Denmark, is inherited by a different dominant gene, *Rz2* (5,14,23). The number of additional genes conferring resistance remains unclear and needs further research (3).

Three major pathotypes of BNYVV have been reported (11–13). Pathotype A was detected in Greece, the former Yugoslavia, Slovakia, part of Austria, Italy, Spain, parts of France, Belgium, the Netherlands, England, Turkey, Kazakhstan, China, Japan, and the United States (13). Pathotype B has been observed in Germany and the upper Rhine Valley in France. Pathotype P has so far been found only in the region near Pithiviers, France (10) and East Anglia in the UK (7). Pathotypes A and B consist of four genomic RNAs (9,21,22), and pathotype P contains a fifth RNA. Experimental evidence has shown that BNYVV isolates containing the fifth RNA are highly virulent and can infect partially resistant beet varieties (26). Sequences of RNA-5 of the European and the Japanese sources are related, but differ by 37 point mutations and 20 insertion/deletion mutations (10). The different BNYVV pathotypes can be distinguished by means of restriction fragment length polymorphism (RFLP) and single-strand conformation polymorphism (SSCP) analyses of reverse transcription–polymerase chain reaction (RT-PCR) products (12,13). SSCP is a powerful tool for the detection of viral genome differences. When the plus and minus strands of a double-stranded DNA are separated by heat treatment, they attain metastable sequence-specific folded structures. The particular electrophoretic mobilities and single nucleotide exchanges can be detected in nondenaturing polyacrylamide gels (19).

In 2002–03, several sugar beet fields with a BNYVV-resistant cultivar Beta 4430 R (*Rz1rz1*) in the Imperial Valley of California were observed with severe rhizomania symptoms. In this research, the resistance-breaking BNYVV isolates in Imperial Valley were isolated and the host ranges and the pathotype of these isolates were determined.

MATERIALS AND METHODS

Soil treatment × cultivar tests. Soil samples collected from various locations were assayed for virus reaction in greenhouse tests. Soil samples used consisted of autoclaved potting soil, BNYVV-infested soil from Spence Field, Salinas, CA, and soil from Imperial Valley Rockwood 158 field (IV-BNYVV soil). Soil samples were mixed in equal parts with autoclaved sand to facilitate ease of root removal of test plants at harvest. Greenhouse benches were disinfected with 10% sodium hypochlorite prior to use. Pots were new 280-ml Styrofoam cups with holes provided in the bottom for drainage. Pots were placed in sterilized plastic saucers spaced on greenhouse benches to avoid contamination by splashing water. After cups were filled with appropriate soil samples, they were drenched with fungicides metalaxyl (Apron 25W) at 0.2 g/liter and PCNB (Terraclor 75W) at 0.25 g/liter to control damping-off and root rot caused by *Pythium* spp. and *Rhizoctonia* spp. Approximately 100 sugar beet seeds were layered on top of each pot and covered with sterilized sand to a depth of approximately 1 cm. Seeds were watered with gentle misting as needed. Following emergence, overhead watering was discontinued and water was added to the saucers directly as needed to prevent wilting. Greenhouses were maintained at 21 to 32°C without supplement light. Samples were harvested at 5 weeks postemergence. Sugar beet cultivars used in experiments resistant to rhizomania included Beta 4430 R (*Rz1rz1*), KWS Angelina (*Rz1rz1*+*Rz2rz2*), experimental hybrid 1927-4H5 (*Rz1rz1*+WB), and a triploid rhizomania-susceptible Beta 6600 (*rz1rz1rz1*). For the soil × cultivar trials, pots were arranged on greenhouse benches in a randomized complete block

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design with three replications and four subsamples for each treatment. Roots from these pots were harvested and tested for BNYVV as described below.

Enzyme-linked immunosorbent assay (ELISA). Samples were prepared by washing roots of seedlings from each pot to remove soil. Root tissue (0.2 g from each root mass) was placed in sample extraction bags containing 2 ml of extraction buffer (0.05 M phosphate-buffered saline, pH 7.2, 0.5% Tween 20, 0.4% dry milk powder) and homogenized with a hand-held roller press (Agdia, Inc., Elkhart, IN). Expressed sap (100 µl per well) was added to duplicate wells of a microtiter plate. Each plate also contained controls including sap from BNYVV-infected beet roots, healthy beet roots, leaf tissue from *B. macrocarpa* plants systemically infected with BNYVV, and leaf tissue from healthy *B. macrocarpa*.

Double antibody sandwich ELISA (4) was used to assay BNYVV. Purified IgG made to BNYVV (1 mg/ml) was used to coat microtiter plates at a 1:1,000 dilution. Alkaline phosphatase-conjugated anti-BNYVV IgG was added to wells (1:1,000 dilution). Alkaline phosphatase substrate (Sigma Chemical Co., St. Louis, MO) was used at a ratio of 5 mg:8.3 ml of substrate buffer. Absorbance readings (A_{405nm}) were made 1 h after the addition of substrate using a Bio-Tek EL312e microplate reader (Winooski, VT). ELISA values 3 times greater than the healthy mean were considered positive (28). Analysis of variance was run to determine statistical differences among treatment means.

Virus isolates. Root samples from Imperial Valley soil in pot culture were ground 1:5 (wt/vol) in 0.1 M phosphate buffer, pH 7.0, using autoclaved mortars and pestles. *Chenopodium quinoa* Willd. plants were mechanically inoculated with the suspension containing a small amount of Celite by means of a cotton swab. Resulting local lesions were subinoculated to *C. quinoa*. Subsequent local lesions were isolated and freeze dried.

Host range. Selected host plant species used for inoculations were held in the dark for 16 to 24 h prior to inoculation. Test plants were mechanically inoculated with local lesions of each BNYVV isolate as above. Inoculated plants were maintained for symptom development in a greenhouse under natural light with a temperature range of 21 to 32°C. Symptoms on test plants were assessed weekly.

RT-PCR. Viral RNA of each BNYVV isolate was extracted from purified virion preparations using the RNeasy Mini Kit (QIAGEN Inc., Valencia, CA). The extracted viral RNA was used as a template for RT-PCR. Primer pairs specific for BNYVV-RNA-5 were synthesized based on available sequence in GenBank (accession no. D63759). The downstream primer 5R (5′ CACAAGGCTCACGCACA 3′) was complementary to nucleotides 243 to 259, and the upstream primer 5F (5′ AAATTCAAAGTACTTTCATATTGTAC 3′) was identical to nucleotides 1 to 26. First strand cDNA and PCR procedures were conducted as described previously (18). BNYVV-positive controls were P-pathotype (from Pithiviers, France) and A-pathotype (from Salinas, CA).

SSCP. RT-PCR products using 20-mer oligonucleotide primers (see below) designed to amplify BNYVV RNAs 1 and 2 (12) were denatured by heating for 5 min at 70°C in an equal volume of formamide containing 20 mM EDTA, 0.1% bromophenol blue, and 0.1% xylene cyanol and immediately cooled on ice. Samples were analyzed by electrophoresis in a 10% polyacrylamide–bisacrylamide gel (29:1). The 0.8-mm-thick gel was run at 200 V constant voltage for 6 h at 4°C in a mini slab unit (Idea Scientific, Corvallis, OR) with 1× TAE (40 mM Tris-acetate, pH 8.0, 1 mM EDTA). After electrophoresis, gels were silver-stained as described by Bassam et al. (2). The primer pairs used for BNYVV-RNA-1 were 5′ CACAGGTTCCGTGAGTATAA 3′, representing nucleotides 6130 to 6149, and 5′ ACAATTAGTTCAATAATTAT 3′, complementary to nucleotides 6652 to 6671 (accession no. X05147). Primers for BNYVV-RNA-2 were 5′ TGAATTATAAGGCCGGTTCT 3′, representing nucleotides 2791 to 2810, and 5′ CGGTACAAGACTTACTAACT 3′, complementary to nucleotides 3242 to 3261 (accession no. X04197). PCR products from RNA-1 (nt 6130 to 6671) were purified using QIAquick Gel Extraction Kit (QIAGEN) and sequenced by a commercial company (MCLAB, South San Francisco, CA). Sequences were analyzed by the software program DNAMAN (Lynnon BioSoft, Vaudreuil, Quebec, Canada).

RESULTS AND DISCUSSION

The rhizomania-resistant cultivars grown in BNYVV-infested soil from Salinas, CA, did not become infected, and all three resistant cultivars were significantly different ($P \leq 0.05$) from the susceptible cultivar Beta 6600 according to relative ELISA values but were not significantly different among themselves (Table 1). These results suggested that *Rz1* conditioned resistance to BNYVV pathotype A. In contrast, when grown in Imperial Valley BNYVV-infested soil, all resistant cultivars became infected with BNYVV and showed high ELISA values, although the cultivar Angelina with two genes for resistance (*Rz1rz1, Rz2rz2*) had a significantly lower ELISA value than the susceptible check (Table 1). These results correspond to what recently has been observed in the field under IV-BNYVV where Angelina and other cultivars with resistance gene *Rz2* or both *Rz1* and *Rz2* had a better survival rate and higher yield (R. T. Lewellen, *unpublished data*). These results suggest that even though defeated by IV-BNYVV, *Rz1* may still provide some protection, and *Rz2* with or without *Rz1* may provide an intermediate level of protection or resistance. For the soil × cultivar test, replication means for ELISA values were very similar and not significantly different. All cultivars grown in sterilized soil were nearly equal to the relative ELISA value of 1.0. Significant differences ($P \leq 0.01$) occurred for sources of infested soil and cultivars. The mean relative ELISA absorbance values for different sources of soil across cultivars were: sterilized = 1.0, Salinas BNYVV = 2.6, and Imperial Valley BNYVV = 5.8, with LSD (0.05) = 2.30. The relative means for the cultivars across soils were: Beta 6600 = 4.6, Beta 4430R = 2.8, Angelina = 2.1, and 1972-4H5 = 3.1, with LSD (0.05) =1.95. Significant interactions occurred between cultivar Beta 6600 (susceptible cultivar) and all other resistant cultivars when contrasted under the Salinas and Imperial Valley BNYVV-infested soils (Table 1). Under both BNYVV soil treatments, ELISA values for Angelina with two factors for resistance (*Rz1* and *Rz2*) were lower than either Beta 4430 R or 1927-4H5 with the single allele *Rz1* for resistance. Under high initial inoculum levels and optimum environmental conditions for rhizomania, disease development may appear to break down partially resistant

Table 1. Enzyme-linked immunosorbent assay (ELISA) A_{405nm} values of sugar beet cultivars grown in Imperial Valley soil compared with Salinas *Beet necrotic yellow vein virus* (BNYVV) infested soil and sterilized check

Beet cultivar (resistance genes/alleles)	Salinas soil	Imperial Valley soil	Sterilized soil
Beta 6600 (*rz1rz1rz1*)	5.1[v] (+)[w] ab[x]	7.6 (+) a	1.0 (–) d
Beta 4430 R (*Rz1rz1*)	2.1 (–) cd	5.4 (+) ab	1.0 (–) d
KWS Angelina (*Rz1rz1* + *Rz2rz2*)[y]	1.2 (–) d	4.0 (+) bc	1.1 (–) d
USDA 1927-4H5 (*Rz1rz1* + WB[z])	2.2 (–) cd	6.2 (+) ab	1.0 (–) d

[v] A_{405nm} of sample/A_{405nm} of healthy check.
[w] + or – based upon > 3× healthy check.
[x] Duncan's multiple range test means for all 12 treatment combinations. Means with a letter in common are not significantly different at the 5% level.
[y] *Rz1rz1* and *Rz2rz2* are dominant alleles at two separate loci.
[z] WB = wild beet, with resistance to BNYVV possibly being derived from *Beta vulgaris* subsp. *maritima*.

Table 2. Response of Salinas and Imperial Valley *Beet necrotic yellow vein virus* (BNYVV) isolates on different hosts

Host	Salinas BNYVV	Imperial Valley BNYVV isolates							
		IV-1	IV-2	IV-3	IV-4	IV-5	IV-6	IV-7	IV-8
Beta macrocarpa	S*[z]	S	S	S	S	S	S	S	S
B. vulgaris (Beta 6600)	CLL	–	S	S	S	S	S	–	–
Chenopodium amaranticolor	C/NLL	CLL	NLL	NR	CLL	CLL	C/NLL	CLL	CLL
C. capitatum	CLL	NLL	–	C/NLL	CLL	CLL	S	–	NLL
C. quinoa	CLL	CLL	CLL	CLL	CLL	CLL	CLL	CLL	CLL
Nicotiana benthamiana	S	–	–	S	–	S	S	–	S
N. clevelandii	–	–	–	S	S	–	–	–	S
N. glutinosa	–	–	–	SNLL	–	NLL	–	–	–
Spinacia oleracea	S	–	S	S	S	CLL	S	–	–
Tetragonia expensa	CLL	CR	–	CLL	CLL	CLL	CLL	NLL	–

[z] * CLL: chlorotic local lesions; C/NLL: chlorotic/necrotic local lesions; CR: chlorotic rings; NLL: necrotic local lesions; NR: necrotic rings; S: systemic infection; SNLL: small necrotic local lesions; –: nonhost.

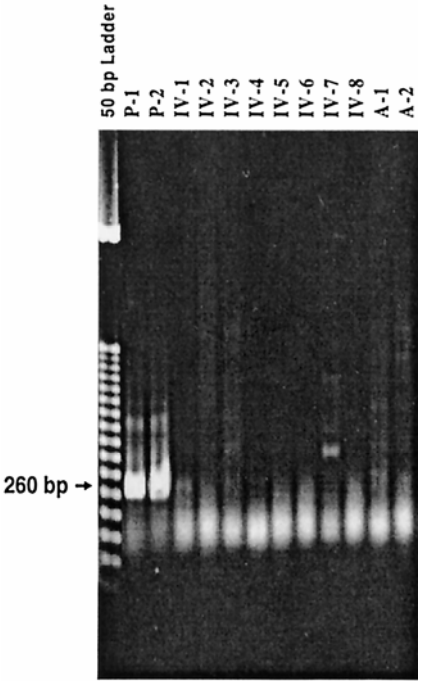

Fig. 1. A 1.5% agarose gel showing amplification products (260 bp) from reverse transcription–polymerase chain reaction (RT-PCR) using *Beet necrotic yellow vein virus* (BNYVV) RNA-5-specific primer pairs. P1 and P2 are P-pathotype from Pithiviers, France; A1 and A2 are A-pathotype from Salinas, CA; IV-1 to IV-8 are BNYVV isolates from Imperial Valley, CA.

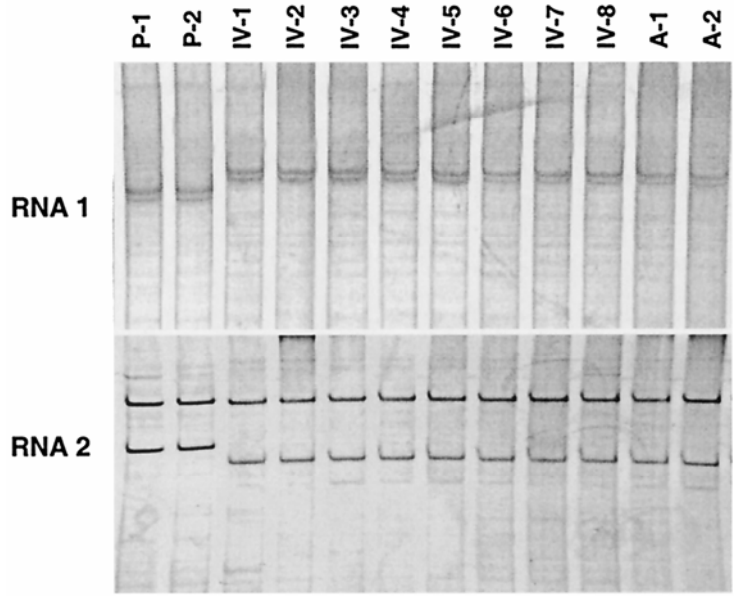

Fig. 2. Single-strand conformation polymorphism analysis patterns of reverse transcription–polymerase chain reaction (RT-PCR) products from *Beet necrotic yellow vein virus* (BNYVV) RNA-1 and RNA-2. P1 and P2 are P-pathotype from Pithiviers, France; A1 and A2 are A-pathotype from Salinas, CA; IV-1 to IV-8 are BNYVV isolates from Imperial Valley, CA. The IV-BNYVV banding patterns from both RNA-1 and RNA-2 are identical to A-pathotype and different from P-pathotype.

cultivars (1). Soil dilution experiments with resistant and susceptible cultivars gave no evidence that the inoculum level affected the reaction of rhizomania-resistant cultivars (data not shown). The most important results from soil × cultivar experiments showed that resistant cultivars in the Imperial Valley had been compromised and that ELISA results correlated with field observations under commercial conditions that the *Rz1* allele had been defeated.

Single local lesion isolates of IV-BNYVV inoculated to a range of host plants identified eight isolates of BNYVV from Imperial Valley that were different from Salinas BNYVV based on host reactions (Table 2). Both IV-BNYVV and Salinas BNYVV showed systemic infection in *B. macrocarpa*. However, after mechanical inoculations on sugar beet, the Salinas BNYVV produced chlorotic local lesions, whereas IV-BNYVV isolates resulted in either no infection or systemic infection. On *C. capitatum,* Salinas BNYVV showed chlorotic local lesions and IV-BNYVV isolates showed a wide range of reactions from nonhost to chlorotic local lesions, necrotic local lesions, and/or systemic infections. It has been rare for BNYVV to produce systemic infection in sugar beet from mechanical inoculation (15). Most resistance-breaking IV-BNYVV isolates have produced systemic infection in sugar beet from mechanical inoculation with the virus. This change suggests that there is a fundamental difference between Salinas BNYVV and IV-BNYVV isolates. It is not yet known if this difference is related to or coincidental with the increased pathogenicity of IV-BNYVV against *Rz1*. The reaction of IV-BNYVV isolates on these hosts has demonstrated the wide biological variability present in BNYVV in just one sugar beet field.

A fifth BNYVV RNA species has been consistently detected in sugar beet infected with the P-pathotype of BNYVV (10). BNYVV RNA-5 specific primer pairs were used for RT-PCR. None of the IV-BNYVV isolates contained RNA-5. In contrast, pathotypes P-1 and P-2 of BNYVV that possesses RNA-5 produced a 260-bp amplification product, while pathotypes A-1 and A-2 that do not possess RNA-5 did not produce the 260-bp amplicon (Fig. 1). The P-pathotype of BNYVV showed increased aggressiveness against both susceptible and resistant sugar beet cultivars (26). It was important, therefore, to demonstrate that the IV-BNYVV isolates did not contain the fifth RNA species and are not the result of the direct introduction of the pathotype P strains.

SSCP analyses indicated that the banding patterns of IV-BNYVV isolates were

Table 3. Sequence alignment at selected nucleotide positions near the 3′ end of *Beet necrotic yellow vein virus* (BNYVV) RNA-1 for differentiation of A and B pathotypes

	Nucleotide position number									
BNYVV source	**6240**	**6267**	**6312**	**6393**	**6394**	**6465**	**6494**	**6513**	**6535**	**6597**
A-Pathotype*[z]	U	C	U	G	C	C	A	U	G	A
B-Pathotype	C	U	C	A	U	U	G	C	U	G
IV-1	U	C	U	G	C	C	A	U	G	A
IV-2	U	U	U	G	C	C	A	U	G	A
IV-3	U	C	U	G	C	C	A	U	G	A
IV-4	U	C	U	G	C	C	A	U	G	A
IV-5	U	C	C	G	C	C	U	U	G	A
IV-6	U	C	U	G	C	C	A	U	U	A
IV-7	U	C	U	G	C	C	A	U	G	A
IV-8	U	C	U	G	C	C	A	A	G	A
S-89	U	C	U	G	C	C	A	U	G	A
S-02	U	C	C	G	C	C	A	U	G	A

[z] * A-pathotype sequence is based on I10/11 (Italy) and Yu2 (former Yugoslavia), and B-pathotype sequence is based on F2 (France) and Rg (Germany), reported by Koenig and Lennefors (11). IV-1 to IV-8 are BNYVV isolates from Imperial Valley, CA. S-89 and S-02 are BNYVV isolates from Salinas, CA. The nucleotide position numbers correspond to the F2 isolate (accession no. X05147). Nucleotides different from A-pathotype are underlined.

identical to those of BNYVV pathotype A and were different from pathotype P in both amplicons from RNA-1 and RNA-2 (Fig. 2). Sequences of IV-BNYVV and Salinas BNYVV isolates in the region of nt 6130 to 6671 in RNA-1 (nt numbering was based on the sequence of the isolate F2, accession no. X05147) compared with A type from Italy (I10/11) and Yugoslavia (Yu 2) as well as B type from France (F2) and Germany (Rg) (11) revealed that BNYVV isolates from Imperial Valley and Salinas were closely related to A-pathotype (Table 3).

Our experimental results indicate that all eight IV-BNYVV isolates were capable of infecting the three BNYVV-resistant sugar beet cultivars tested (17). These isolates did not contain RNA-5. When combined with SSCP analyses and sequence comparison, these results suggest that the resistance-breaking BNYVV isolates from Imperial Valley may have evolved from existing A-pathotype isolates.

In 2002, three fields in the Imperial Valley were identified with BNYVV isolates that could overcome the resistant cultivars (according to soil tests described above) and in 2003, five additional fields were identified. In 2004, at least 26 fields were suspected of containing the resistance-breaking isolates based on soil tests and commercial variety performance. It is unknown if the increased level of virulence in these fields was due to a single event with subsequent field-to-field virus spread or selection from naturally occurring variation within each field.

The large-scale field planting of resistant cultivars may cause significant selection pressure on the virus leading to partial or total breakdown of resistance (8,20,29). Consequently, the selection of beet cultivars should be assessed, not only against the original A-pathotype, but also against resistance-breaking isolates described here, when considering cultivar choice. Additional sources of resistance with different genetic determinants should also be sought to increase the stability and durability of the resistance.

A search for additional sources of resistance, specifically to the isolates found in the Imperial Valley, will be important for the sugar beet industry. A wide array of *B. vulgaris* germ plasm will be evaluated in Salinas in an attempt to identify resistance to these new isolates of BNYVV. In addition, the industry has established a field plot in situ in the Imperial Valley for evaluating host plant resistance to these emerging isolates.

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